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(54) Title: YEASTS CONTAINING MULTIPLE MAMMALIAN CYTOCHROMES FOR DEGRADING COMPLEX CHEMICAL MIXTURES

(57) Abstract

The present invention relates to the functional expression of multiple, eukaryotic cytochrome P450s in yeast cells, genetic constructs for effecting such expression and methods employing these yeast cells and/or the recombinant enzymes so produced for metabolizing chemically-complex cytochrome P450 substrates. The invention relates, in an important respect, to the use of such recombinant yeast cells to affect metabolism of crude oils. In particular, the invention relates to the ability of the engineered yeast, due to the multiple, eukaryotic cytochrome P450s cloned therein, to degrade various constituents of crude oil.

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YEASTS CONTAINING MULTIPLE MAMMALIAN CYTOCHROMES FOR DEGRADING COMPLEX CHEMICAL MIXTURES

The present invention relates to the functional expression of multiple, eukaryotic cytochrome P450s in yeast cells and populations of yeast, genetic constructs for affecting such expression and methods employing these yeast cells or populations of yeasts and/or the recombinant enzymes produced by such cells or populations of cells for metabolizing chemically-complex cytochrome P450 substrates, particularly complex chemical mixtures. The invention relates, in an important respect, to the use of such recombinant yeast cells and populations of such cells to affect metabolism of crude oils and related products. In particular, the invention relates to the ability of the recombinant yeasts or populations of yeasts, to express functional, multiple, eukaryotic cytochrome P450s cloned therein and to degrade various constituents of petroleum.

Among the diverse biological systems known which are capable of degrading complex chemicals, certain eukaryotic enzymes appear to be the most efficient and to possess the broadest substrate range. The enzymes responsible for this ability of eukaryotes are the cytochrome P450 proteins (generally referred to as "P450s"). These proteins utilize electrons from reduced nucleotide phosphates (NADPH or NADH) to activate molecular oxygen. The reducing equivalents are relayed to the cytochrome P450 via one or

two additional but distinct proteins (e.g., reductases) that together form a short electron-transport chain.

The general reaction catalyzed by cytochrome P450 is as follows:

$$RH + NADPH^{+} + H + O_2 \rightarrow ROH + NADP^{+} + HOH$$

where RH is a suitable P450 substrate and ROH is its hydroxylated product. In higher organisms, the microsomal system which catalyzes this reaction is composed of a cytochrome P450 reductase and a family of cytochrome P450 isozymes associated with microsomal membranes of the cell.

The ability of these enzymes to activate molecular oxygen and subsequently to insert one oxygen atom into a substrate has been exploited by organisms for many purposes. Consequently, there are dozens of different cytochrome P450 molecules even in a single organism. Each P450 enzyme has a different substrate specificity, but, unlike the situation with most enzymes, the substrate specificity of a single cytochrome P450 may be quite broad.

The substrates of cytochrome P450 are lipophilic molecules frequently containing multiple ring structures. Some major functions of cytochrome P450 proteins include the metabolism of drugs and other foreign compounds, steroidogenesis and bile acid production from cholesterol, and ω-oxidation of fatty acids and prostaglandins (for reviews on cytochrome P450, see, White and Coon, 1980; Adesnik and Atchison, 1986; Nebert and Gonzalez, 1987; and Black and Coon, 1986).

Despite numerous sequence similarities, cytochrome P450 proteins are diverse and it is, therefore, helpful to categorize them. The first major dichotomy is between

eukaryotic and prokaryotic P450s. The bacterial P450s form a large class of soluble proteins, in contrast to the eukaryotic enzymes, which are all integral membrane proteins.

Among the eukaryotic P450s, many vertebrate sequences are known. Thus, for instance, enzymes from rats have been purified and characterized (Dignam and Strobel, 1977) as have various other P450 isozymes (Saito and Strobel, 1981; Lau and Strobel, 1982). The enzymes are found in two cellular locations, the mitochondria and the endoplasmic reticulum. The mitochondrial enzymes catalyze the earliest steps in the conversion of cholesterol into steroid hormones. The endoplasmic-reticulum enzymes are referred to as microsomal P450s.

The microsomal P450s can be divided into two groups on the basis of their substrates. The microsomal P450s act on endogenous substrates. They are involved in steroid-hormone biosynthesis and metabolism and in the metabolism of fatty acids and prostaglandins and may be referred to as endogenous P450s. The majority of sequenced microsomal P450s, however, act on compounds foreign to the host cell such as drugs and petroleum products. They constitute part of a detoxification system and may be referred to as the xenobiotic-metabolizing P450s.

In fact, a large percentage of the known P450 sequences are xenobiotic-metabolizing cytochrome P450s capable of drug and xenobiotic metabolism. These proteins can be induced up to 70-fold by the administration of drugs or xenobiotics. The proteins fall into two families that are induced by two different classes of drugs, the phenobarbital (PB) family and the 3-methylcholanthrene (MC) family. Figure 1 lists the cytochrome P450 sequences grouped into protein families (nomenclature of Dayhoff,

1979). In general, the wide capability of mammalian cytochromes to degrade complex chemical mixtures matches the composition of crude oil and petrochemical wastes.

Cytochrome P450 genes are arranged in multigene families that together constitute a gene super family. The actual number of P450 genes in mammals is not known, but a lower limit may be estimated on the basis of molecular-hybridization studies. Two non-cross reacting probes have indicated a minimum of 15 genes in the PB family (Atchison and Adesnik 1983; Mizukami et al. 1983). Five rat and six rabbit P450 sequences are known, so approximately one-third of the genes in these species have been sequenced.

The MC family also has a number of genes. Two sequences each are known in rats, mice, and humans. Three are present in rabbits. The glucocorticoid-inducible $P450_{PCN}$ (also called $P450_p$) shows six to eight hybridization bands (Gonzalez et al. 1985). P450 C21 is present as two genes in each of cattle, mice, and humans, with only one gene each functional in mice and man (Chung et al. 1986a). The LA_ω family contains two or three genes (Hardwick et al. 1987). There are at least six additional P450 proteins in mammals: P450 17α, $P450_{scc}$, P450 11β, P450 aromatase, 25-hydroxy vitamin D_3 1α-hydroxylase, and cholesterol 26 hydroxylase. Therefore, the minimum number of P450 genes is 33, with the possibility that there are others (for a more complete discussion of the genetics of cytochrome P450, see Adesnik and Atchison 1986).

Eukaryotic P450s have been cloned and expressed in bacterial expression systems. For instance, Porter et al. (1987) relates to the expression of a functional mammalian cytochrome P450 reductase in E. coli. Japanese Patent 2,016,976 relates to recombinant cytochrome "b5" reductase expression in E. coli. Unfortunately, however, the present

understanding in the art is that many eukaryotic cytochrome P450s cannot be expressed functionally in bacteria (Cullin, et al., 1988).

Eukaryotic cytochrome P450s have been expressed in eukaryotic expression systems, as well. U.S. Patent Application Serial No. 7/303,898 of Battula and U.S. Patent Application Serial No. 7/058,387 of Gelboin et al. both relate to the use of recombinant retro-viruses for expressing P450 cytochromes in mammalian cells. COS1 cells (Zuber, et al., 1986) and virally infected eukaryotic cells (Battula, et al., 1987; Asseffa, et al., 1989) have been used as hosts for the heterologous expression of P450 molecules, yet each has limitations to their usefulness as expression systems. In particular, the eukaryotic cell lines do not provide a cell population which has the potential for being sustained in an environment outside strictly controlled laboratory conditions. Thus, even fewer systems for cells expressing cytochrome P450s are available which can be disseminated outside controlled laboratory conditions.

It is known that the eukaryotic yeasts possess their own P450s and the genes for these yeast enzymes have been cloned and expressed. Kiessling et al., E. German Patent 271,339, relates to the isolation of a cytochrome P450 gene from the yeast Candida maltosa that hydroxylates alkanes. Azari and Wiseman (1982) demonstrated the presence of cytochrome P-448 in S. cerevisiae related to an isozyme of rat P450 c which possesses benzo(a)pyrene hydroxylase activity. In rats, this particular P450 enzyme is induced by oral administration of crude oil (Kahn and Rahimtula, 1987). Loper and colleagues (1985) suggested that yeasts containing the activity for aromatic hydrocarbon degradation may be used in seeding experiments for clean-up of toxic wastes in the environment. Other related yeast

proteins have also been recombinantly expressed in yeast. For instance, Japanese Patent 2,000,451 relates to the cloning of a yeast cytochrome P450 reductase in E. coli.

Yeasts are known to be capable of heterologous expression of eukaryotic P450s from other eukaryotes, as well. For instance, Japanese Patent Applications 63,044,888 and 62,104,583 both relate to chimeric mammalian (rat liver) genes expressed in yeast which produce protein fusions between cytochrome P450IA1 (also called "P450MC" from yeasts) and a reductase or P450IA1 and P450d, respectively. The latter fusion protein results from part of IA1 (i.e. HR2 region of P450MC) being substituted with part of P450d ("the same region of P450d"). Thus, these abstracts report the expression in yeast of hybrid proteins, not found in nature, with multiple cytochrome P450 activity. Japanese Patent 1,047,380 relates to a yeast strain that produces bovine cytochrome P450 XVIIA1 from a recombinant plasmid. This particular cytochrome reacts with progesterone or pregnenolone. Japanese Patent Application (62,019,085) relates to the expression of rat liver cytochrome P450 reductase in Saccharomyces cerevisiae. This cytochrome is expressed from a recombinant plasmid using an alcohol dehydrogenase promoter. U.S. Patent 4,766,068 issued to Hiroko et al. relates to the expression of rat liver cytochrome P450 IA1 ("P450MC") in Saccharomyces cerevisiae from a recombinant plasmid using the yeast alcohol dehydrogenase promoter.

However, man-made chimeric enzymes are known to suffer instability problems. This is especially true where such cloned enzymes are subjected to the rigors of growth in a natural environment away from intense selection techniques found in cultured conditions such as in bioreactors and fermentors.

Moreover, while a single isozyme of P450 is able to address a number of slightly different compounds due to its ability to bind a variety of substrates -- a very unusual ability in the world of enzymes -- single cytochromes or chimeric enzymes derived therefrom are not particularly suited to initiating the degradation of complex mixtures of organic compounds such as those found in petroleum waste. The complexity of chemical mixtures typically encountered in petroleum wastes is not suited to the use of single enzymes which are not as versatile as naturally occurring systems in this regard.

Means are needed for: (1) simply and rapidly inducing stable cytochromes capable of initiating the degradation of the complex mixtures found in a given petroleum mixture by oxidizing varied groups of compounds which are the substrates of different cytochrome P450s; (2) expressing the combinations of cytochromes suitable for the given petroleum product; (3) expressing such combinations of cytochromes in situ in an environment free of intense selection for the genetic construction encoding the selected cytochromes; (4) achieving this expression in an organism capable of safe, intentional environmental release; and (5) potentially improving the degradative capabilities of the naturally-occurring cytochromes.

The present invention seeks to overcome one or more problems and deficiencies in the prior art by providing populations of recombinant yeast cells that encode at least two distinct P450 enzymes thereby assisting in the biodegradation of complex mixtures of chemical compounds such as hydrocarbon mixtures, e.g., petroleum product mixtures. However, because of the wide variation in crude oil components, it would be desirable to have a number of P450 enzymes involved in the biodegradative process, each one able to take care of a variety of substrates.

Therefore, the present invention relates to the creation of a number of yeast strains and/or populations of such yeast cells, which are optimized in their degradation of alkane, asphaltene, aromatic, etc., compounds.

Due to the existence of numerous disadvantages with current systems for the expression of biologically active cytochrome P450s, there exists a continuing need for the development of novel systems which can be used to produce biologically active cytochrome P450 enzymes. Since yeasts are eukaryotes, they possess an endoplasmic reticulum and as such represent a better host for functioning of the mammalian cytochrome P450s than do the prokaryotes of the prior art approaches. There is a particular need for yeast or other simple eukaryote expression systems for expressing biologically active eukaryotic cytochrome P450s which incorporate the advantages of yeast cell expression. The development of novel technology as is disclosed herein which addresses one or more of these disadvantages would have broad research and commercial applications including drug development and environmental clean up.

In one part, the present invention relies on the fact that yeast, as opposed to other commonly used eukaryotic cellular systems, have the ability to survive in a variety of environmental situations. There is a need to combine the ability of the endogenous P450 system of yeast to degrade petroleum products with the ability of mammalian P450 systems which has been genetically engineered into the same strain or strains of yeast. The substrate specificity of mammalian P450 enzymes will improve the native oil-degradative ability of the yeast strains substantially. The potential of the engineered yeast for use in detoxification of wastes in the environment would have many advantages over systems currently used.

Overcoming these limitations is achieved by the present disclosure in part by designing yeast strains which contain at least pairs of stable P450 isozymes with different specific activities for the different substrates found in crude oil. Furthermore, a method is disclosed in the present invention for eliminating a particular petroleum waste from a given site by analyzing the crude mixture to determine the relative amounts of alkanes, asphaltenes, and aromatic compounds present, and then to introduce the strains of yeast which can best degrade those components.

By using yeasts and multiple P450s cloned therein, the present invention overcomes at least some of the disadvantages of the prior art. First, no requirement is made for man-made chimeric enzymes. This is especially advantageous since environmental release of such organisms away from intense selection techniques found in cultured conditions seriously undermine the usefulness of chimeric proteins. Additionally, the present invention takes advantage of the ability of single isozymes of P450 to attack a number of slightly different compounds. This is in part due to the ability of such cytochrome P450s to bind a variety of substrates while including selected combinations of such enzymes to complement the complex mixture of compounds in a petroleum product. Since yeast represent a host which potentially may be distributed outside the laboratory environment, a more flexible approach to environmental degradation of complex chemical mixtures is possible.

In particular, the advantages of the invention over the prior art are achieved by providing: (1) a simple and rapid means for inducing stable, naturally-occurring cytochromes capable of initiating the degradation of the complex mixtures found in a given petroleum mixture by

inducing such enzymes in eukaryotes and cloning the genes therefor; (2) a means for coordinately expressing the combinations of cytochromes suitable for the given petroleum product; (3) a means for expressing such combinations of cytochromes in situ in an environment free of intense selection for the genetic construction encoding the selected cytochromes; (4) achieving this expression in an organism or population of organisms capable of safe, intentional environmental release; and (4) potentially improving the degradative capabilities of the naturally-occurring cytochromes.

Figure 1: P450 Genes and Their Products

Figure 2: Construction of Yeast Vectors Containing Mammalian Cytochrome P450b.

Figure 3: CO Difference Spectra Comparing Yeast Vectors With and Without Mammalian Cytochrome P450b.

In order to achieve the advantages mentioned above, the invention provides a population of stably transformed yeast cells recombinantly expressing at least a first and a second biologically active eukaryotic cytochrome P450 enzyme. The yeast cell population may be an homogenous population in which each yeast cell constituting the population is a virtually identical sister cell of a single parental cell containing the same genetic information as the parent cell. In this case, the genetic material contained within each sister cell in the population will encode the necessary genes to express at least two of the recombinant eukaryotic cytochrome P450s.

The population may, on the other hand, represent a heterologous population of yeast cells. In this instance, while it is still possible that a single cell line may

contain all of the genetic information necessary to express at least two recombinant eukaryotic cytochrome P450s, the invention provides for a mixed population in which single recombinant eukaryotic P450s may be encoded by different cells within the population.

The enzymes expressed within the population are chosen for their capacity to degrade at least two distinct chemical components of an admixture such as petroleum mixtures. Thus, the enzymes recombinantly established in the populations of the invention may be selected to degrade any of the general classes of compounds which are typically found in such complex hydrocarbon mixtures such as short chain alkanes, long chain alkanes, cyclic alkanes, substituted alkanes, simple aromatic hydrocarbons, complex aromatic hydrocarbons, substituted aromatic hydrocarbons or the like.

Where the recombinant yeast cell population of the invention represent the progeny of a single parent yeast cell, the recombinant enzymes may be encoded in more than one plasmid or other genetic vector. In many instances, however, it will likely be preferred to clone both recombinant enzymes into a single plasmid which is then transformed into a single progenitor cell. Where there is a single plasmid encoding both of the recombinant cytochrome P450s, the enzymes may be operatively linked each to a separate promoter sequence. However, in many instances, and in particular where it is advantageous to coordinately control the expression of the two recombinant P450s, the genes encoding the separate enzymes will be operatively linked to a single promoter sequence. Such gene hook-ups may resemble a coordinately controlled operonic system where a first gene is downstream of a second gene and each is controlled by a promoter sequence immediately upstream of the first gene. Alternatively, and

preferred in many instances is a hook-up where a bi-directional promoter is operatively linked on either of its flanking regions to one or the other of the two recombinant P450s. Such a bi-directional promoter is provided by the invention by example as the GAL1-GAL10 promoter sequence. Other promoters that may be substituted include the alcohol dehydrogenase promoter of yeast.

The population of yeast cells of the invention is constructed to possess first and second eukaryotic enzymes for the degradation of selected first and second substrates. This is most readily achieved by selecting each enzyme from any of the different known cytochrome P450 families possessing eukaryotic P450 members. These families are represented in Figure 1. Thus, for example, the first enzyme may be chosen from the cytochrome P450 I, II, III, IV, VI, XIA, XIB, XVII, XIX, XXI or XXVI family. Similarly, the second enzyme is chosen from the same or a different one of these families. In any case, the selection of the individual enzyme or the family of enzymes from which it is to be chosen is dictated by the constituents of the complex chemical admixture which is to be attacked. The invention is particularly concerned with the design of such populations capable of degrading admixtures comprising petrochemical components, select components of which are degradable by a eukaryotic cytochrome P450 enzyme.

The invention further provides a process for constructing a population of stably transformed yeast cells recombinantly expressing at least two distinct biologically active eukaryotic cytochrome P450 enzymes capable of degrading different chemical components of an admixture. This process is initiated by inducing expression of at least two cytochrome P450 enzymes by administering at least two different components of the complex chemical admixture

to eukaryotic subject animals, typically rats or other laboratory animals, in order to induce transcription of mRNA encoding the cytochrome P450 produced by the animal to metabolize the administered compound.

The test animals are then sacrificed and the mRNA is collected from their tissues (typically liver) and cDNA corresponding to the mRNA is isolated. Where sequence information is available (preferably DNA sequence information although amino acid sequence information will suffice), the cDNA gene sequences encoding the cytochrome P450s are then isolated by hybridization to short oligonucleotide probes by techniques known well to those of skill in the art. The isolated cDNA sequences are then cloned in yeast expression vectors and inserted into yeast cells.

In preferred embodiments, the invention also provides a stably transformed yeast cell which expresses at least two independent biologically active eukaryotic cytochrome P450 enzymes. These active enzymes are encoded in recombinant cytochrome P450 genes and are operatively associated with those necessary expression elements which are capable of effecting yeast transcription and translation of said genes such as yeast-compatible ribosome binding sites, spacer regions, transcription terminators, and promoters capable of promoting expression of the genes.

The genetic constructions of the invention may be placed on any number of vehicles known well to those of skill in the art so long as such vehicle allows the cloned gene to be expressed in a yeast cell. Thus, plasmids such as pMAC561, pBINK may be useful in this regard.

It will be appreciated by those of skill in the relevant art that the population of yeast cells may, in

addition to the cytochrome P450 enzymes, possess other recombinant enzymes. In particular, it may be advantageous to provide an enzyme normally associated with the electron transfer apparatus of the membrane bound eukaryotic P450s such as a eukaryotic cytochrome P450 reductase.

As a result of the yeast cell populations and process for producing such populations, the invention further provides a method for the simultaneous production of at least two biologically active eukaryotic cytochrome P450 enzymes. The method comprises preparing the yeast cell population in accordance with the previously discussed techniques and then growing the cell population under conditions appropriate to effectuate expression of the cytochrome P450 enzymes.

In some cases, the conditions under which the yeast cell population is grown will be under highly controlled, laboratory or bioreactor conditions. Under these conditions, the chief goal will be to maximize production of the enzyme without undue concern for permanently establishing the population in the laboratory environment. However, as a principal advantage to expression in other eukaryotic cells which do not grow under the harsher conditions of the natural environment, yeast populations may also be established, in some cases relatively permanently, in the natural environment or at least in environments where other eukaryotic cell lines would not prosper. Thus, the methods of the invention provide for initiating the growing of the yeast cell population by releasing the yeast cell population into an open environment or natural environment such as a pond, lake, field, tilled soil, or other situs in which the introduction of such population is desired. It is suggested that such environments might be of particular

interest where the environment has been contaminated by a petrochemical.

A method for the metabolism of eukaryotic cytochrome P450 substrates is also provided which method comprises preparing a yeast cell population which expresses at least two biologically active recombinant cytochrome P450 enzymes in accordance with previously discussed techniques. Again, the expressed enzymes are selected for their capability to metabolize selected substrates of interest. Then, the method calls for subjecting the substrates to the enzymes so produced under conditions conducive to metabolizing the substrates. This may be achieved with the whole cells of such yeast populations. Alternatively, it may be achieved where the enzymes are at least partially purified from said yeast cell population prior to subjecting the substrates to the enzymes. In some cases it may be preferable to at least partially maintain the integrity of the cell membranes with which the cytochrome P450s and reductases are associated.

A DNA segment is also provided in the invention which comprises at least two gene sequences encoding at least two biologically active eukaryotic cytochrome P450 enzymes capable of degrading distinct chemical components of an complex admixture. This DNA segment may further comprise cytochrome P450 enzyme genes modified to allow yeast expression of the encoded cytochrome P450 enzymes in a biologically active form. This is typically achieved by incorporating yeast-compatible ribosome binding sites, spacer regions, transcription terminators, and promoters capable of effecting yeast expression of said genes.

In more specific terms, in order to develop a simple method for generating suitable combinations of mammalian cytochrome P450s, a variety of alkanes may be used to treat

rats. Observations of the induction of messenger RNA for isozymes specific for the different alkanes will then be carried out.

With these and other data designs of a number of yeast strains will be enabled which optimize the ability to address the bioremediation of any given crude oil sample. Thus, for example, for crude mixtures containing high levels of polyaromatic compounds, yeast expressing P450 isozymes IA1 and IA2 may be used. For crude mixtures containing high levels of alkanes, use of a combination of yeast strains expressing P450 isozymes IIC11 and IIE1, as well as the other forms listed in Figure 1 with their specificities for crude oil components is advisable.

In certain instances, it may be best to use a combination of different yeast strains in varying proportions. In addition, use of the cloned rat NADPH P450 reductase expressed in plasmid pRS315 will be advisable in certain instances. Both plasmids may be carried in the same yeast strain YPH 499, or another similar GAL+ strain, such as GRF167 altered to contain the trp1 and leu2 mutations.

Crude oil mixtures will be analyzed by the company or field which owns the oil. The composition will vary but the classes of compounds usually are reflected in broad solubility classes. Typically, pentane extraction is used to separate out all soluble compounds from the tarry asphaltenes. The pentane extract is further extracted by hexane, benzene or other solvents, into fractions. These gross fractions will typically be characterized further with a variety of analytical techniques (e.g. gas chromatography, high performance liquid chromatography, mas spectrometry). The contents fall into general

categories, alkanes and simple and complex aromatic compounds.

The choice of which P450 or P450s to use will be made, depending on either the content of crude oil or crude oil fraction using the activity/induction correlation shown in Table 1. The data presented in Table 1 are derived from the prior art literature and from the results of unpublished induction studies disclosed herein in which rats were treated with a series of hydrocarbons and assayed for P450 forms expressed.

Table I

P450 Form		Crude oil components
b	IIB1	alkanes
c	IA1	polycyclic aromatic hydrocarbons
f	IIC7	simpler aromatic hydrocarbons
d	IA2	simple substituted benzenes
j	IIE1	short chain alkanes
Laω	IVA1ω	long chain alkanes
h	IIC11	alkyl substituted benzenes

Investigations were initiated during the making of the present invention to test the ability of certain cytochrome P450 isozymes to degrade crude petroleum wastes when expressed in the yeast. Initially, studies were conducted in order to determine whether S. cerevisiae with a PEP4 mutation could express cytochrome P450IIB1 at a constitutively high level when controlled by the alcohol dehydrogenase promoter. A cDNA clone for P450IIB1 was cloned into the yeast expression vector pMAC561 (McKnight and McConaughy 1983). In order to achieve this, the yeast

strain 20B-12 Jones 1977 containing a PEP4 mutation Jones 1977 was transformed with a plasmid pBINK, which contains the P450IIB1 cDNA gene oriented 5′ to 3′ in functional linkage with the yeast alcohol dehydrogenase promoter; or with a plasmid pKNIB, with the gene in the opposite orientation functionally linked to the same promoter (Figure 2). The plasmids were transformed into yeast by the lithium acetate transforation procedure such as that described in *Current Protocols in Molecular Biology*, Vol. 2 (Wiley Interscience, 1989).

A reduced CO difference spectrum, which spectrum is capable of detecting the presence of the cytochrome P450, was performed on the yeasts using 2 x 10^9 cells/ml in 0.1M potassium phosphate buffer, pH 7.5. These spectra were achieved by methods known well to those of skill in the art. The cDNA gene for P450IIB1 was expressed in these yeast cells from the alcohol dehydrogenase promoter. This was demonstrated by an increase in absorbance at 454 nm in a CO difference spectrum (Fig. 3). The expression was no different when the yeast were grown with or without oxygen. No cytochrome P450 was detected in cells containing the vector alone.

Additionally, the transformed yeast strains were tested for their ability to grow on oil-containing plates. This was accomplished by the procedures of Walker and Colwell (1976). All of the strains grew poorly or not at all on plates containing crude oil as the major carbon source (results not shown). This lack of growth occurred whether or not minor carbon sources such as minimal concentrations of glycerol were initially present in the medium.

In order to construct a vector which could be used to express a given pair of cytochrome P450s at a high level,

the bi-directional GAL1-GAL10 promoter was investigated. Mammalian cytochrome P450IA1 (P450c) was expressed from a GAL promoter in the plasmid pOLV1. This plasmid was constructed using the GAL1-GAL10 promoter from the plasmid pBM150 (Johnston and Davis, 1984) inserted into the yeast-E. coli shuttle vector pRS316 (Sikorski and Hieter, 1989). Specifically, the BamHI-EcoRI fragment of pBM150 containing the GAL1-GAL10 promoter was cloned into the BamH1-EcoRI site of the multiple cloning site of the vector pRS316 to form pOLVI.

Several clones of IA1 were obtained by amplification using the polymerase chain reaction technique (PCR). The IA1 isozyme was previously cloned by Hines et al. (1985). Using the DNA sequence data published by these researchers, the present researchers designed oligonucleotides and then used these oligonucleotides in the amplification of cDNA from β-naphthoflavone-treated rat liver.

Specifically, in order to obtain a cDNA clone of P450IA1 for expression in yeast, RNA was isolated from the livers of male rats treated with β-naphthoflavone. RNA isolated from these cells using standard guanidine HCl techniques known well to those of skill in the art, was then reverse transcribed and subjected to PCR amplification using oligonucleotides designed to hybridize to the cDNA at the 5′ end of the gene SEQ. ID NO. 1: (5′-GATCATGCCTTCTGTGTATGG-3′) and to the opposite strand at the 3′ end of the gene SEQ. ID NO. 2: (5′-AGTCTAAGCCTGGAGATGCTGAGG-3′). The amplified DNA was subjected to electrophoresis and was removed from the gel. The fragment thus amplified and purified was then cloned into pBLUESCRIPT KS(+) (Stratagene) by blunt end ligation, and confirmed by restriction analysis.

In a preliminary study, a small number of cells of each strain were lysed and subjected to SDS page and western analysis using antibody prepared to rat P450IA1. P450IA1-PCR clones C1, C33, C35, and C47 all showed some cross-reactivity to the antibody.

Determination of the sequence of the P450IA1 clones obtained by PCR amplification was carried out. The BamHI-HindIII restriction fragments from pBLUESCRIPT containing P450IA1-PCR were cloned into bacteriophage M13 for sequencing for three of the clones. Approximately 300 base pairs were sequenced from either the N-terminus or the C-terminus of the resulting protein, depending on orientation of the clone in the original pBLUESCRIPT.

P450IA1 cloned by PCR amplification of rat hepatic RNA demonstrated certain mutations from the sequences known in the prior art. In certain cases, such mutations will not cause deleterious changes in the resulting protein. However, it will be understood by those of skill in the art that standard DNA sequencing of the resulting DNA may be achieved to verify that a DNA fragment contains an accurate sequence for a given cytochrome. This may be further confirmed by assaying for the respective activity as noted above.

In order to determine whether P450IA1 clones obtained by PCR amplification and cloning could be expressed by pOLV in S. cerevisiae, yeast strain YPH499 containing the IA1-PCR clones C1, C33, C35, or C47 was grown in minimal medium with galactose as the sole carbon source. The cells were harvested in stationary phase and a reduced CO difference spectrum was obtained from each strain (as described above). No significant difference was found in the amount of absorbance in the 450-460 nm range. However, there was a difference in peak position for one of the clones

indicating the desired activity was present in this clone. Cells expressing the cloned cytochrome were shown to remain viable but not to grow on media containing crude petroleum.

It is possible to select for strains of yeast containing these plasmids for those with improved metabolism of petroleum and petroleum products. This may be accomplished by introducing random mutations into the transformed cells and selecting mutants with increased growth on petroleum.

Rat P450 reductase may be incorporated into the yeast strains of the invention on a separate plasmid, pRS315 (Sikorski and Hieter, 1989). Incorporation of rat reductase along with the other clones of the invention may be advisable since its presence in the cell would potentially improve the function of the expressed rat P450s. Such inclusion is, however, not a requirement for the successful use of the yeast strains of the invention.

Cytochrome P450IA1, the enzymatic activities of which enzyme have been shown to be induced in rat liver and kidney following feeding of the rats with Prudhoe Bay crude oil (Khan and Rahimtula, 1987), was used for studies herein. A genomic clone encoding P450IA1 (Hines, et al., 1985) was used to probe a cDNA library from rat liver (Clontech Laboratories) for a complete cDNA clone of P450IA1 by standard techniques (Maniatis et al., 1982) as shown above.

The cDNA clone was placed in the yeast shuttle vector YEp24 (Botstein et al., 1979). YEp24 is able to replicate under antibiotic selection in E. coli by virtue of its pBR322 sequences, and can replicate under uracil selection in yeast due to the 2 micron episomal DNA sequences and the presence of the yeast URA3 gene.

The heterologous P450 gene was expressed from the URA3 promoter. This vector has the advantage of a high copy number so that expression of plasmid-borne genes will occur at a higher level than that of a chromosomal gene using the same promoter. Furthermore, unlike many high-expression promoters (ADH1, GAL1; PH05), the URA3 promoter is less stringently regulated and will permit transcription under a variety of growth conditions. Finally, YEp24 has previously been used successfully to express another type of cytochrome P450 (lanosterol demethylase) in yeast (Kalb, et al., 1986).

The DNA manipulations were performed in E. coli and the plasmid was transformed into yeast (Sherman et al., 1985) under selection for uracil prototropy. Expression of cytochrome P450IA1 was confirmed by Western analysis of crude extracts of yeast by the method of Loper et al. (1985). As shown above, antibodies to rat cytochrome P450IA1 were used to identify the enzyme's presence in yeast. Expression of the mammalian cytochrome P450 was measured by an elevation in benzo(a)pyrene hydroxylase activity over the same yeast strain which did not contain the heterologous enzyme.

Yeast strains carrying the genetic constructions of the invention will be altered to enhance the likelihood that they will not survive in the environment without oil. In one such embodiment, rho minus yeast (which yeasts are unable to carry out respiration for energy) which are also blocked in various glucose metabolism steps may be used. In order to address alkane degradation as well as polymeric degradation, some of metabolic steps must remain. Thus, each strain to be disabled will have to be selectively so disabled depending on the (relatively) specific degradation task to which the strain will be put.

The mutagenesis of the selected strains while growing on crude oil plates will be accomplished. This will be followed by replica-plating to glucose or glycerol-containing plates and detection of colonies unable to grow. By so selecting those that are unable to grow, ability of the strains to grow on certain readily found carbon sources in the environment will be diminished.

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* * * * * * * *

The present invention has been described in terms of particular embodiments found or proposed to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

CLAIMS:

1. A population of stably transformed yeast cells recombinantly expressing at least first and second biologically active eukaryotic cytochrome P450 enzymes, the enzymes capable of degrading, respectively, at least a first and second chemical component of an admixture.

2. The population of claim 1, wherein the enzymes degrade short chain alkanes, long chain alkanes, cyclic alkanes, substituted alkanes, simple aromatic hydrocarbons, complex aromatic hydrocarbons or substituted aromatic hydrocarbons.

3. The population of claim 1, wherein the yeast cells are progeny of a single parent yeast cell.

4. The population of claim 1, wherein the enzymes are cloned into a single plasmid.

5. The population of claim 4, wherein the enzymes are operatively linked to a single promoter sequence.

6. The population of claim 5, wherein the promoter sequence is the GAL1-GAL10 promoter sequence.

7. The population of claim 1, wherein the first and second enzymes are selected from any of the different cytochrome P450 families represented in Figure 1.

8. The population of claim 1, wherein the admixture comprises petrochemical components degradable by a eukaryotic cytochrome P450 enzyme.

9. A process for constructing a population of stably transformed yeast cells recombinantly expressing at least first and second biologically active eukaryotic cytochrome P450 enzymes, the enzymes degrading, respectively, at least a first and second chemical component of a mixture, comprising:

(a) inducing expression of at least two cytochrome P450 enzymes by administration of at least two different components of said mixture to eukaryotes;

(b) preparing cDNA corresponding to mRNA isolated from said eukaryotes;

(c) isolating the gene sequences encoding said cytochrome P450s from said eukaryotes;

(d) cloning said gene sequences in yeast expression vectors; and

(e) inserting said expression vectors into yeast cells.

10. A stably transformed yeast cell which expresses at least two independent biologically active eukaryotic cytochrome P450 enzymes, said enzymes encoded in recombinant cytochrome P450 genes having associated expression elements capable of effecting yeast transcription and translation of said genes, said elements comprising yeast-compatible ribosome binding sites, spacer regions, transcription terminators, and at least one promoter capable of promoting expression of the genes.

11. The population of claim 1, further comprising a eukaryotic cytochrome P450 reductase.

12. A method for the production of at least two biologically active eukaryotic cytochrome P450 enzymes in a recombinant yeast cell population comprising preparing the yeast cell population in accordance with claim 10 and growing said cell population under conditions appropriate to effectuate expression of the cytochrome P450 enzymes.

13. The method of claim 13 where growing of the yeast cell population is accomplished by releasing the yeast cell population into an open environment.

14. The method of claim 14 where the open environment is contaminated by a petrochemical.

15. A method for the metabolism of a first and second eukaryotic cytochrome P450 substrate comprising preparing a yeast cell population which expresses at least two biologically active recombinant cytochrome P450 enzymes in accordance with claim 10, wherein said expressed enzymes are capable of metabolizing said substrates, and subjecting said substrates to the enzymes so produced under conditions effective to metabolize said substrate.

16. The method of claim 16, wherein said enzymes are at least partially purified from said yeast cell population prior to subjecting the substrates to the enzymes.

17. The method of claim 17, wherein said recombinant eukaryotic cytochrome P450 substrates are subjected to yeast cell membrane fractions which comprise the enzymes.

18. The method of claim 16 wherein said substrates comprise petrochemical components of an admixture.

19. A DNA segment comprising at least two gene sequences encoding at least first and second biologically active eukaryotic cytochrome P450 enzymes, the enzymes capable of degrading, respectively, at least a first and second chemical component of an admixture.

20. The DNA segment of claim 20 further comprising at least two cytochrome P450 enzyme genes modified to allow yeast expression of the encoded cytochrome P450 enzymes in a biologically active form, said genes incorporating yeast-compatible ribosome binding sites, spacer regions, transcription terminators, and promoters capable of effecting yeast expression of said genes.

Family	Locus Symbol	Protein Name	Trivial Name	Species and Source[a]	References	
I	CYP1A1	IA1	c	Rat	D	Sogawa et al. (1984)
					R	Yabusaki et al. (1984)
					D	Hines et al. (1985)
			P_1	Mouse	R	Kimura, S. et al. (1984b)
					R	Kimura, S. et al. (1987b)
			P_1	Human	R	Jaiswal et al. (1985a)
					D	Jaiswal et al. (1985b)
					R	Quattrochi et al. (1985)
					D	Kawajiri et al. (1986)
			form 6	Rabbit	R	Okino et al. (1985)
					R	Kagawa et al. (1987)
			IA1	Trout	RD	Heilmann et al. (1988)
	CYP1A2	IA2	d	Rat	R	Kawajiri et al. (1984)
					D	Sogawa et al. (1985)
					P	Haniu et al. (1986)
			P_3	Mouse	R	Kimura, S. et al. (1984a)
					R	Kimura, S. et al. (1984b)
			P_2	Mouse	R	Kimura and Nebert (1986)
			P_3	Human	R	Jaiswal et al. (1986)
					R	Jaiswal et al. (1987)
			form 4	Human	R	Quattrochi et al. (1985)

FIG. 1A

			LM4	Rabbit	RD	Quattrochi et al. (1986)
					P	Fujita et al. (1984)
					R	Okino et al. (1985)
					P	Ozols (1986)
					R	Kagawa et al. (1987)
IIA	CYP2A1	IIA1	a1	Rat	R	Nagata et al. (1987)
	CYP2A2	IIA2	a2	Rat	R	Matsunaga et al. (1988)
	CYP2A3	IIA3	a3	Rat	R	Kimura, S. et al. (1989)
			15α	Mouse	R	Squires and Negishi (1988)
			P450(1)	Human	R	Phillips et al. (1985a)
			IIA3	Human	R	Yamano, S. et al.
IIB	CYP2B1	IIB1	b	Rat	R	Fujii-Kuriyama et al. (1982)
					R	Gotoh et al. (1983)
					P	Yuan et al. (1983)
					R	Phillips et al. (1983)
					R	Affolter and Anderson (1984)
	CYP2B2	IB2	e	Rat	R	Fujii-Kuriyama et al. (1982)
					D	Mizukami et al. (1983)

FIG. 1B

Family	Locus symbol	Protein name		Species and Trivial name	Source[a]	References
					P	Yuan et al. (1983)
					R	Phillips et al. (1983)
					R	Affolter and Anderson (1984)
	CYP2B3	IIB3	IIB3	Rat	R	Labbé et al. (1988)
	CYP2B4	IIB4	LM2	Rabbit	P	Heinemann and Ozols (1983)
					P	Tarr et al. (1983)
			p54		R	Komori et al. (1988)
			B0		R	Gasser et al. (1988)
			P-450$_1$, b15		R	Komori et al. (1988)
			B1		R	Gasser et al. (1988)
			b46		R	Komori et al. (1988)
	CYP2B4P		(pseudogene)	Rabbit	D	Zaphiropoulos et al. (1986)
	CYP2B5	IIB5	b52	Rabbit	R	Komori et al. (1988)
			B2		R	Gasser et al. (1988)
			HP1		R	Komori et al. (1988)
	CYP2B6	IIB6	LM2	Human	R	Miles et al. (1988)

FIG. 1C

	CYP2B7	IIB7		Human	R	Yamano, S. et al.
	CYP2B8	IIB8		Human	R	Yamano, S. et al.
	CYP2B9	IIB9	pf26	Mouse	R	Noshiro et al. (1988)
	CYP2B10	IIB10	pf3/46	Mouse	R	Noshiro et al. (1988)
IIC	CYP2C1	IIC1	PBc1	Rabbit	R	Leighton et al. (1984)
	CYP2C2	IIC2	PBc2, K	Rabbit	R	Leighton et al. (1984)
					D	Govind et al. (1986)
			PHP2		R	Imai et al. (1988)
	CYP2C3	IIC3	PBc3	Rabbit	R	Leighton et al. (1984)
			3b		P	Ozols et al. (1985)
	CYP2C4	IIC4	1-88	Rabbit	R	Johnson et al. (1987)
			PBc4		R	Zhao et al. (1987)
	CYP2C5	IIC5	form 1	Rabbit	R	Tukey et al. (1985)
	CYP2C6	IIC6	PB1	Rat	R	Gonzalez et al. (1986a)
			pTF2		R	Friedberg et al. (1986)

FIG. 1D

Family	Locus symbol	Protein name		Species and Trivial name	Source	References
					R	Kimura, H. et al. (1988)
	CYP2C6P	(pseudogene)		Rat	R	Kimura, H. et al. (1988)
	CYP2C7	IIC7	f	Rat	R	Gonzalez et al. (1986a)
			pTF1		R	Friedberg et al. (1986)
	CYP2C8	IIC8	form 1	Human	R	Okino et al. (1987)
			IIC2		R	Kimura, S. et al. (1987a)
			mp-12, mp-20		HumanRGed et al. (1988)	
	CYP2C9	IIC9	IIC1	Human	R	Kimura, S. et al. (1987a)
					R	Yasumori et al. (1987)
					R	Meehan et al. (1988a)
			mp-4		R	Ged et al. (1988)
	CYP2C10	IIC10	mp	Human	R	Umbehauer et al. (1987)
			mp-8	Human	R	Ged et al. (1988)
	CYP2C11	IIC11	h, M-1, 16α	Rat	R	Yoshioka et al. (1987)
					D	Morishima et al. (1987)
					R	Zaphiropoulos et al. (1988)

FIG. 1E

	CYP2C12	IIC12	i, 15β	Rat	R	Zaphiropoulos et al. (1988)
	CYP2C13	IIC13	g	Rat	R	McClellan-Green et al. (1988)
	CYP2C14	IIC14	pHP3	Rabbit	R	Imai (1987)
	CYP2C15	IIC15	b32-3	Rabbit	R	Imai et al. (1987)
IID	CYP2D1	IID1	db1	Rat	R	Gonzalez et al. (1987)
			CMF2	Rat	R	Ishida et al. (1988b)
	CYP2D2	IID2	db2	Rat	R	Gonzalez et al. (1987)
			CMF2	Rat	R	Ishida et al. (1988b)
	CYP2D3	IID3	db3	Rat	R	Matsunaga, E. et al.
	CYP2D4	IID4	db4	Rat	R	Matsunaga, E. et al.
			CMF3	Rat	R	Ishida et al. (1988b)
	CYP2D5	IID5	db5	Rat	R	Matsunaga, E. et al.
			CMF1b	Rat	R	Ishida et al. (1988b)
	CYP2D6	IID6	db1	Human	R	Gonzalez et al. (1988b)

FIG. 1F

SUBSTITUTE SHEET

Family	Locus symbol	Protein name		Species and Trivial name	Source[a]	References
					R	Gonzalez et al. (1988c)
	CYP2D7	IID7		Human	D	Kimura, S. et al.
	CYP2D8	IID8		Human	D	Kimura, S. et al.
	CYP2D9	IID9	16α	Mouse	R	Wong et al. (1987)
	CYP2D10	IID10	cb	Mouse	R	Ichikawa et al. (1989)
IIE	CYP2E1	IIE1	j	Human	R	Song et al. (1986)
					D	Umeno et al. (1988a)
				Rat	R	Song et al. (1986)
					D	Umeno et al. (1988b)
			3a	Rabbit	R	Khani et al. (1987)
					R	Imai et al. (1988)
					D	Khani et al. (1988)
	CYP2E2	IIE2	IIE2	Rabbit	D	Khani et al. (1988)
IIF	CYP2F1	IIF1		Human	R	Nhamburo et al.

FIG. 1G

IIG	CYP2G1	IIG1	olf1	Rat	R	Nef et al. (1989)
IIH	CYP2H1	IIH1	PB15	Chicken	R	Hobbs et al. (1986)
IIIA	CYP3A1	IIIA1	pcn1	Rat	R	Gonzalez et al. (1985)
	CYP3A2	IIIA2	pcn2	Rat	R	Gonzalez et al. (1986b)
	CYP3A3	IIIA3	HLp	Human	R	Molowa et al. (1986)
	CYP3A4	IIIA4	nf-25	Human	R	Beaune et al. (1986)
			pcn1		R	Gonzalez et al. (1988a)
			nf-10		R	Bork et al. (1989)
	CYP3A5	IIIA5	pcn3	Human	R	Aoyama, T. et al.
	CYP3A6	IIIA6	3c	Rabbit	R	Dalet et al. (1988)
IVA	CYP4A1	IVA1	LAω 1	Rat	R	Hardwick et al. (1987)
	CYP4A2	IVA2	LAω 2	Rat	R	Kimura, S. et al.
	CYP4A3	IVA3	LAω 3	Rat	D	Kumara, S. et al.
	CYP4A4	IVA4	p-2	Rabbit	R	Matsubara et al. (1987)

FIG. 1H

Family	Locus symbol	Protein name		Species and Trivial name	Source*	References
	CYP4A5	IVA5	LAω 1	Rabbit	R	Johnson, E.F. et al.
	CYP4A6	IVA6	LAω 2	Rabbit	R	Johnson, E.F. et al.
	CYP4A7	IVA7	LAω 3	Rabbit	R	Johnson, E.F. et al.
IVB	CYP4B1	IVB1	Lung P450	Human	R	Nhamburo et al.
			form 5	Rat	R	R. Gasser and R.M. Philpot
			form 5	Rabbit	R	R. Gasser and R.M. Philpot
VIA	CYP6A1	VIA1		House fly	R	Feyereisen, R. et al. (1989)
XIA	CYP11A1	XIA1	scc	Human	R	Chung et al. (1986b)
					D	Morohashi et al. (1987a)
				Cow	R	Morohashi et al. (1984)
					P	Chashchin et al. (1986)
XIB	CYP11B1	XIB1	11β	Cow	R	Chua et al. (1987)
					R	Morohashi et al. (1987b)
XVII	CYP17	XVIIA1	17α	Cow	R	Zuber et al. (1986)
				Human	R	Chung et al. (1987)
					D	Picado-Leonard and Miller

FIG. 11

						(1987)
					R	Bradshaw et al. (1987)
					D	Kagimoto et al. (1988)
				Pig	R	Chung et al. (1987)
				Rat	R	Nishihara et al. (1988)
					R	Namiki et al. (1988)
				Chicken	R	Ono et al. (1988)
XIX	CYP19	XIXA1	arom	Human	R	Simpson et al. (1987)
					R	Chen, S. et al. (1988)
					R	Corbin et al. (1988)
				Chicken	R	McPhaul et al. (1988)
XXI	CYP21A1	XXIA1	c21A	Mouse	D	Chaplin et al. (1986)
			c21	Cow	D	Chung et al. (1986a)
					R	John et al. (1986)
					R	Yoshioka et al. (1986)
			c21	Pig	P	Haniu et al. (1987)
	CYP21A1P	(pseudogene c21A)		Human	D	Higashi et al. (1986)
					D	White et al. (1986)
	CYP21A2	XXIA2	c21B	Human	D	Higashi et al. (1986)
					D	White et al. (1986)
					R	Matteson et al. (1987)

FIG. 1J

Family	Locus symbol	Protein name	Species and Trivial name	Source[a]	References
	CYP21A2P	(pseudogene c21B)	Mouse	D	Chaplin et al. (1986)
XXVI	CYP26	XXVIA1 26-ohp	Rabbit	R	Andersson, S. et al.
	CYP51	P450LI 14DM	*S. cerevisiae*	D	Kalb et al. (1987)
				D	Ishida et al. (1988)
			C. tropicalis	D	Chen, C. et al. (1988)
	CYP52	P450LII alk	*C. tropicalis*	D	Sanglard and Loper (1989)
	CYP101	P450CI cam	*Ps. putida*	P	Haniu et al. (1982)
				D	Unger et al. (1986)
	CYP102	P450CII BM-3	*B. megaterium*	D	Ruettinger, R.T. et al.

aD, DNA; R, CDNA derived from RNA; P, protein sequence.

FIG. 1K

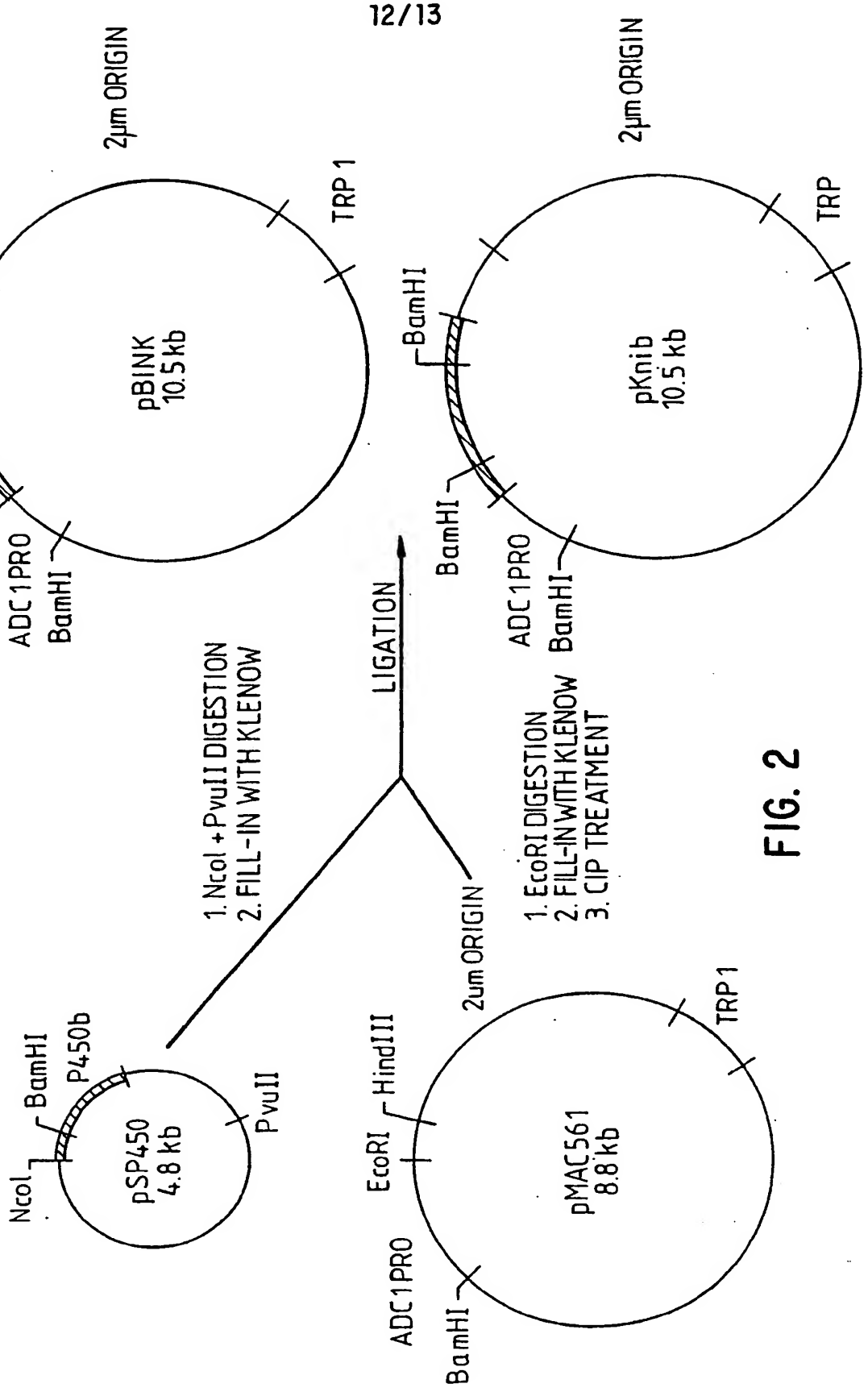

FIG. 2

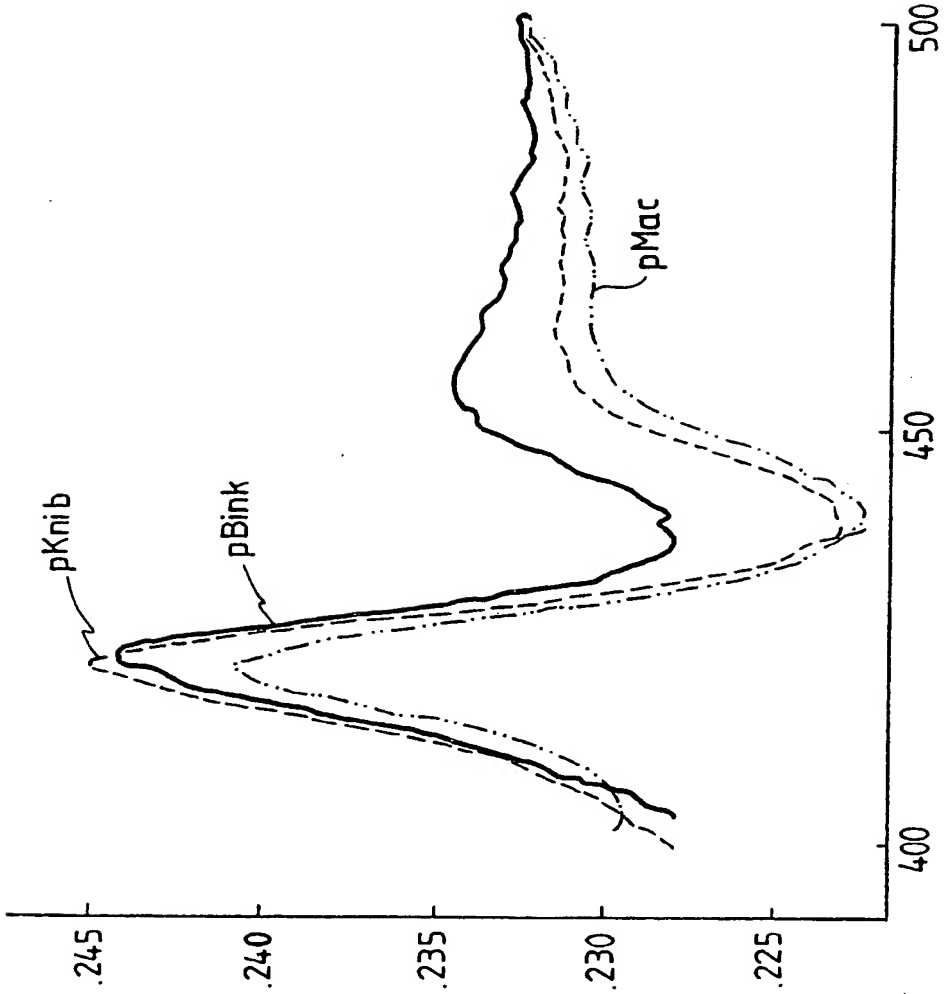

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09033

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/19, 15/52; C12P 1/02
US CL :435/255, 172.3, 69.1; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/255, 172.3, 69.1; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A, 0,360,361 (Slijkhuis et al.) 28 March 1990, see entire document.	1-20
Y	EP, A, 0,132,309 (Davis et al.) 30 January 1985, see entire document.	1-20
Y	Canadian Journal of Physiology and Pharmacology, Volume 65, issued 1987, S. Khan et al., "Effect of a Prudhoe Bay crude oil on hepatic and placental drug metabolism in rats", pages 2400-2408, especially see Abstract.	1-20
Y	Journal of Biochemistry, Volume 101, No. 5, issued 1987, Y. Imai, "Cytochrome P-450 Related to P-450(4) from Phenobarbital-Treated Rabbit Liver: Molecular Cloning of cDNA and Characterization of Cytochrome P-450 obtained by Its Expression in Yeast Cells", pages 1129-1139, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 JANUARY 1993

Date of mailing of the international search report

27 JAN 1993

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